Plant Nutritional Genomics

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Plant Nutritional Genomics

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Preface

A 'textbook' plant typically comprises about 85% water and 13.5% carbohydrates. The remaining fraction contains at least 14 mineral elements, without which plants would be unable to complete their life cycles. These essential mineral elements include six macronutrients – N, K, P, S, Mg and Ca – which are present in relatively large amounts in plant tissues (mg g^{-1} of dry tissue), and several micronutrients, including Fe and Zn, which are present in smaller amounts (μg g^{-1} of dry tissue). Tissue concentrations of these essential mineral elements must be maintained within a certain range, since mineral deficiencies limit growth and crop production, and mineral excesses are toxic. In addition, plants accumulate non-essential and/or toxic mineral elements such as Sr, Na, Cd and Pb, when these are present in the soil.

Understanding plant nutrition and applying this knowledge to practical use is important for several reasons. First, nutrient deficiencies in crop production can be remedied by the application of fertilisers. However, fertiliser use incurs direct financial costs to the farmer and indirect costs to society. Indirect costs include the consumption of energy during the production, transport and application of fertilisers, and the depletion of finite natural resources. Further, since many crops do not recover fertilisers efficiently, unrecovered nutrients can pollute adjacent natural habitats, leading to a decline in species biodiversity. An understanding of plant nutrition allows fertilisers to be used more wisely. Second, the nutritional composition of crops must be tailored to meet the health of humans and livestock. Over three billion people worldwide do not receive adequate amounts of mineral elements such as Ca, Zn, Fe and Se in their diets, due to the low mineral content of many staple food crops. An understanding of plant mineral nutrition allows this 'hidden hunger' to be sated. Third, many regions of the world are currently unsuitable for crop production due to soil salinity, acidity, or contamination with toxic elements such as heavy metals or radionuclides. An understanding of plant nutrition can be used to develop strategies either for the remediation/restoration of this land, or for the cultivation of novel crops.

The application of knowledge of plant nutrition can be achieved through genotypic or agronomic approaches. Genotypic approaches, based on crop selection and/or breeding (conventional or GM), have recently begun to benefit from technological advances, including the completion of plant genome sequencing projects. This book is intended to provide an overview of how *plant nutritional genomics,* defined as *the interaction between a plant's genome and*

its nutritional characteristics, has developed in light of these technological advances, and how this new knowledge might be usefully applied.

In the first section of the book, the molecular physiology of the uptake, transport, and assimilation of the major plant mineral nutrients are reviewed. Françoise Daniel-Vedele and Sylvain Chaillou (INRA-Versaille) have described how genomics can help researchers to understand the mechanisms of uptake and utilisation of N (Chapter 1). Similarly, Malcolm Hawkesford (Rothamsted Research) has reviewed the genes impacting on the uptake, transport and assimilation of S (Chapter 4). Molecular aspects of P transport have been described by Kashchandra Raghothama (Purdue) (Chapter 5) and Philip White (Warwick HRI) has provided a comprehensive overview of the genetics of Ca accumulation (Chapter 3). Sabine Zimmermann and Isabelle Chérel (INRA-Montpellier) have described the molecular biology and regulation of K^+ uptake (Chapter 2) and the first section concludes with a review of sodium (Na^+) tolerance and Na^+ transport (Chapter 6) by Huazhong Shi (Texas) and colleagues. In the second section, techniques to enable the study of plant nutritional genomics are discussed, including the use of high throughput ionomic profiling, by Brett Lahner and David Salt (Purdue) (Chapter 7), and transcriptional profiling, by Frans Maathuis (York), and Anna Amtmann (Glasgow) (Chapter 8). The use of natural genetic variation to study plant nutrition in both model and crop species is reviewed by Dick Vreugdenhil and colleagues (Wageningen) (Chapter 9) and by Matthias Wissuwa (IRRI) (Chapter 10). The final section of the book provides insights into how plant nutritional genomics might be useful in an applied context. Depending upon your viewpoint, these chapters illustrate either (i) how far we have come in a short period of time or (ii) how far we have yet to travel. In Chapter 11, Toby Kiers and Ford Denison (Davis) have provided a thought-provoking insight into the long-term sustainability of crop nutrition. Michael Grusak (Baylor College of Medicine, Houston) and Ismail Cakmak (Sabanci University, Istanbul) have described international efforts to improve the mineral composition of crops in Chapter 12. The book concludes with an in-depth discussion by Steven Whiting, Alan Baker (Melbourne) and colleagues of the role of plants in the restoration or remediation of sites contaminated with heavy metals (Chapter 13).

This book is aimed at researchers and professionals, together with postgraduate students. However, we hope that the material will also stimulate advanced undergraduate students and those interested in the application of this knowledge. We thank the authors for their contributions to this volume, and Graeme MacKintosh and David McDade (Blackwell Publishing) for helping to solicit and edit the material. We would also like to thank John Hammond (Warwick HRI) for his comments on certain chapters. Finally, we thank our families for their continued support.

Martin R. Broadley
Philip J. White

1 Nitrogen

Françoise Daniel-Vedele and Sylvain Chaillou

1.1 Introduction

Nitrogen is a major component of amino and nucleic acids. The main sources of nitrogen (N) for plants are nitrate (NO_3^-) and ammonium (NH_4^+), although plants are also able to exploit organic N sources including amino acids, amides and urea. Plant species from a small number of plant families (e.g. the Fabaceae) are able to use molecular dinitrogen (N_2) as an N source through symbioses with N-fixing bacteria. Compared to C, H and O, which account for 90% of plant dry matter, the N content of plants is low, comprising 1–5% (Mengel & Kirkby, 1987; Marschner, 1995; Heller *et al*., 1998), although N levels of up to 7.5% have been observed in the shoots of *Arabidopsis* (Loudet *et al*., 2003). Proteins and NO_3^- account for 50% and 40% of total shoot N, respectively (Loudet *et al*., 2003), and free amino acids account for 5–10% of total shoot N. Nitrate can be translocated in the xylem sap, although it is relatively phloem-immobile. In contrast, free amino acids circulate readily between roots and shoots through the xylem and phloem, and growing organs supply amino acids to this pool (Cooper & Clarkson, 1989). Ammonium occurs in the xylem sap, but only at low concentrations, for example 0.05 to 1 mM in pea or oilseed rape (Rochat & Boutin, 1991; Schjoerring *et al*., 2002). Nitrate accumulation in the vacuoles of leaf cells can reach high concentrations (40–70 mM), and thus vacuolar NO_3^- can provide a reserve of N for the plant, and it may also contribute to the overall osmotic pressure of the leaf, and therefore to plant turgor (Chaillou & Lamaze, 2001). An osmotic role for NO_3^- is supported by the observation that an *Arabidopsis* mutant, deficient in a NO_3^- transporter (the *chl1* mutant), has a reduced stomatal opening which correlates with reduced NO_3^- accumulation in its guard cells (Guo *et al*., 2003). Nitrate has a further role in water relations since it can promote water transport from roots to shoot, possibly by regulating the expression of aquaporin genes (Limami & Ameziane, 2001; Wang *et al*., 2001). In addition to metabolic and turgor-related roles, NO_3^- also has a signalling role, for example through the induction of genes involved in N and C metabolism (Crawford & Forde, 2002). Ammonium cannot replace NO_3^- in its osmotic or signalling functions and it is toxic at the cellular level (von Wiren *et al*., 2001). However, NH_4^+ is a reduced form of N, which can be rapidly assimilated into amino acids without an energy-costly reducing step. It is therefore paradoxical that NO_3^- is the preferential N source for most plant species, since a complex

reduction pathway requiring two enzymes, (nitrate reductase, NR, and nitrite reductase, NiR) and energy equivalent to 15 moles of ATP per mole of NO_3^-, is required for assimilation of NO_3^- (Fig. 1.1). It is possible that this paradox reflects an adaptation of plants to the mineralisation of organic N, which is prevalent in the majority of aerobic soils of the world, particularly in temperate regions, which ultimately leads to the dominance of NO_3^- as an N source in most soils.

The amount of N necessary for a plant to complete its life cycle varies greatly between species. Some plants are less N demanding than others. For example, many non-agricultural plant species can thrive under conditions of low N whilst high-yielding agricultural species have a high N demand. The genetic basis of differing N requirements between species is still unknown, although quantitative genetics could offer promising insights into the phenomena (Glass & Siddiqi, 1995; Hirel *et al*., 2001; Loudet *et al*., 2003). Further, the N demand of a plant varies according to its developmental stage. For example, N demand is high during vegetative growth and decreases during the reproductive phase, which corresponds with the remobilisation of reserves accumulated as NO_3^-, amino

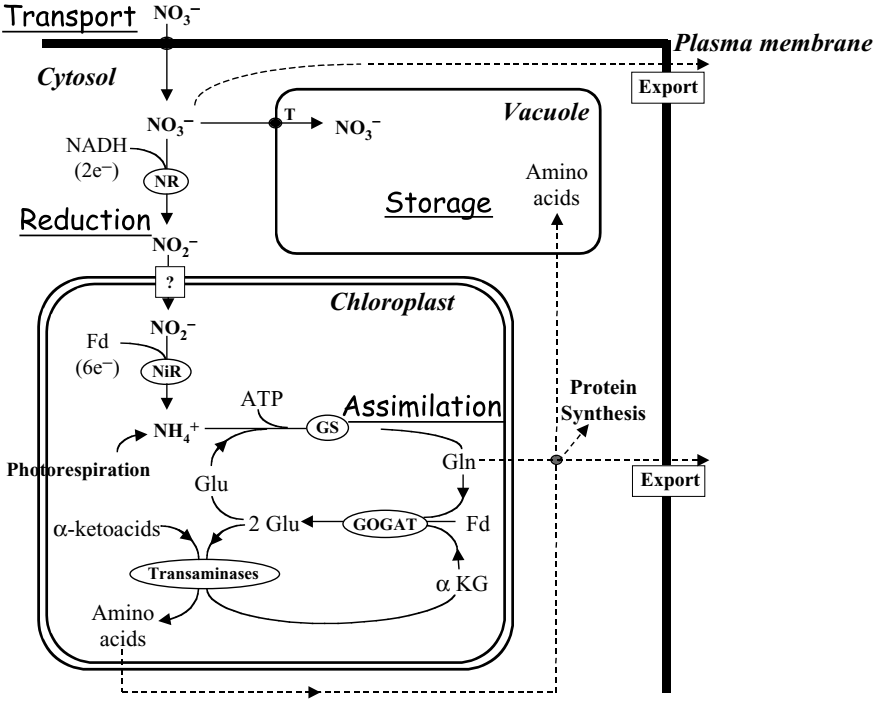

Figure 1.1 The N-assimilation pathway. Different cellular compartments are indicated in italic whilst the different steps of the pathway are underlined.

acids or proteins in different organs during the vegetative growth. Knowledge of the chronological changes in N demand throughout the plant developmental cycle has led to improvements in N-fertilisation practices, allowing reductions in the use of N fertilisers, especially in cereal production. Further, a greater understanding of N-assimilation pathway has allowed crop physiologists to design methods to test the N status of a plant, for example by measuring the NO_3^- content of xylem sap. This has allowed crop-based N demands to be determined and fertiliser applications adjusted accordingly. Reducing N-fertiliser inputs in crop production can reduce leaching losses of NO_3^-, which therefore minimises the pollution of water courses, and can reduce unnecessary financial costs (Meynard *et al*., 2002).

Knowledge of the N composition of plants is also important in food production. For example, wheat grain for use in bread production must have protein content in excess of 12%. Conversely, the protein content of barley grain for use in beer production must not exceed 10%. A further issue on the N composition of plants is the debate on the safe levels of NO_3^- in fresh produce. This has led to intense debates between producers, researchers and the wider public. For example, it is possible that eating salad leaves such as lettuce (*Lactuca sativa*) or spinach (*Spinacia oleracea*) may be hazardous to human health if the NO_3^- content exceeds 2500 mg NO_3^- kg^{-1}f. wt, according to official European standards, whilst cattle may be poisoned by formation of methaemoglobin if the NO_3^- content of fresh herbage exceeds 1500 mg NO_3^- kg^{-1} f. wt (Van Diest, 1986).

It is, therefore, clear that the study of N in plants is important in the context of sustainable agriculture, food quality and food safety. *This chapter will show how genomics can help researchers understand the mechanisms of N uptake and transport. It will review the genomics approaches used to study the enzymes responsible for N assimilation, and describe the search for new genes and their target functions. The use of this information to create new cultivars with improved N-use efficiency will be discussed.*

1.2 Ammonium and nitrate uptake and transport within the plant

Both anionic and cationic forms (NO_3^- and NH_4^+, respectively) of inorganic N are usually available in natural soils but their relative concentrations can vary dramatically. In temperate climates with well-aerated soils, NH_4^+ concentrations are very low, due to rapid nitrification. Conversely, NH_4^+ is the main source of N in acidic or waterlogged soils, and under mixed NO_3^-/NH_4^+ nutritional conditions NH_4^+ is often the preferential form of N taken up by the root system (Dubois & Grenson, 1979; Glass & Siddiqi, 1995; Gazzarrini *et al*., 1999). Nitrate and NH_4^+ concentrations can vary by three or four orders of magnitude in agricultural soils (Wolt, 1994). With certain exceptions, higher

plants are able to cope with these variations and have developed uptake systems for each ion. These systems differ in their specificity and affinity, and their functioning is regulated at the level of gene expression (transcriptional) as well as post-transcriptionally.

Inside root cells, NO_3^- and NH_4^+ may be redirected towards different targets. Nitrate can be stored in the vacuole, where it may become the main source of N when the external supply becomes limiting (der Leij *et al*., 1998), or may contribute to the general osmoticum. It can also be reduced to nitrite (NO_2^-) in the cytosol by nitrate reductase (NR). Finally, it can be redirected out of the root cell either by export to the external medium or by unloading to xylem vessels, from where it can reach the aerial part of the plant (Forde & Clarkson, 1999). All of these NO_3^- or NO_2^- movements require transport across different membranes. Thermodynamic calculations show that NO_3^- transport across the root plasma membrane is an active process (Glass & Siddiqi, 1995). The compartmentation of NH_4^+ is also highly complex, since ammonium is derived from NO_3^- reduction, but most comes from photorespiration, degradation of proteins or transamination reactions. Intriguingly, evidence to challenge the assumption that NH_4^+ concentrations in normal plant tissues is low (Howitt & Udvardi, 2000) has recently been obtained (Britto *et al*., 2001). Further, although it is believed that NH_4^+ generated or absorbed in roots is assimilated immediately, translocation of NH_4^+ from the root to the shoot can occur (Schjoerring *et al*., 2002).

Dissecting the molecular basis of soil-to-plant, or within-plant, fluxes of N has been the challenge for the past decade. The enormous and rapid progress in plant functional genomics has already revealed some of the molecular components of these complex pathways. In this section, we will describe the characteristics of these transport systems, their known molecular components and the regulation of their activities at the physiological and molecular levels.

1.2.1 Ammonium uptake and transport

Net uptake of NH_4^+ by root cells is the difference between influx and efflux. Influx is usually measured using isotopes as $^{13}NH_4^+$ or $^{15}NH_4^+$ during short-term experiments (Clarkson *et al*., 1996). A biphasic pattern of influx is observed for many species such as *Lemna gibba*, rice or *Arabidopsis*. Below external NH_4^+concentrations ($[NH_4^+]_{ext}$) of 1 mM, influx operates via a saturable high-affinity transport system (HATS), whilst a non-saturable low-affinity transport system (LATS) is active at $[NH_4^+]_{ext}$ above 1 mM (Wang *et al*., 1993). The kinetic parameters calculated for the HATS may vary from one species to the other and within the same species depending on environmental conditions (von Wiren *et al*., 2001). This diversity may result from co-existing transporters, each of them being involved in a particular process and showing different kinetic properties. This hypothesis is strengthened by the discovery of a multigenic family potentially encoding several NH_4^+ transporters.

1.2.2 Molecular analysis of ammonium uptake

To identify genes involved in NH_4^+ transport, mutants resistant to methylammonium, a toxic homologue of NH_4^+ which shares the same transporters (Venegoni *et al*., 1997), have been isolated in many species, from yeast (Dubois & Grenson, 1979) and *Chlamydomonas reinhardtii* (Franco *et al*., 1987) to *Nicotiana plumbaginifolia* (Godon *et al*., 1996). Functional complementation of a yeast mutant defective for methylammonium uptake led to the identification of the first NH_4^+ transporter gene from yeast and simultaneously from *Arabidopsis* (Marini *et al*., 1994; Ninnemann *et al*., 1994). From southern blot analysis and, more recently, from the sequenced genome of *Arabidopsis*, the *AtAMT1* gene family can be seen to comprise five homologous members and a more distantly related gene, *AtAMT2*. These encode hydrophobic proteins of 475–514 amino acids which belong to the ammonium transporter (AMT)/methylammonium permease (MEP) family, which are ubiquitous across bacteria, archae, fungi, plants and animals (Saier *et al*., 1999). Deduced amino acid sequences and prediction analyses indicate that an 11 trans-membrane domain is probably present in eukaryotic members of the family, with an outside localisation of the N terminus, which has been experimentally demonstrated for the yeast MEP2 protein (Marini & Andre, 2000). The yeast heterologous expression system has been successfully used to determine the kinetic properties of these proteins. Different substrate affinities (Km) for NH_4^+ were observed among the different AtAMT1 members. Whilst AtAMT1;2 and AtAMT1;3 showed Km values between 25 and 40 μM, AtAMT1;1 had a Km value lower than 0.5 μM (Gazzarrini *et al*., 1999). However, recent studies found no difference between AtAMT1;1 and AtAMT1;2 in their affinity for NH_4^+ (Shelden *et al*., 2001). *AtAMT1;1*, *AtAMT1;2*, *AtAMT1;3* and *AtAMT2* are expressed in roots. Other *AMT* homologues have been cloned from rice – *OsAMT1;1* and *OsAMT2* (Suenaga *et al*., 2003) – and tomato – *LeAMT1;1*, *LeAMT1;2* and *LeAMT1;3* (Lauter *et al*., 1996; von Wiren *et al*., 2000). In tomato, *LeAMT1;1* and *LeAMT1;2* are preferentially expressed in root hairs, thus raising the NH_4^+ uptake efficiency because NH_4^+ is strongly adsorbed to soil constituents. Interestingly, *LeAMT1;3* is preferentially expressed in leaves and the protein exhibits unique features such as a short N terminus when compared to AMT proteins from *Arabidopsis* or rice (von Wiren *et al*., 2000).

1.2.3 Regulation of ammonium uptake: physiological evidence and molecular basis

N uptake by roots is controlled by the N demand of the whole plant linked to the external N availability. For example, a decrease in the $[NH_4^+]_{ext}$ from 1 mM to 0.2 μM led to an adaptative response in rice that simultaneously decreased the Km (from 188 to 32 μM) and increased the maximum influx rate (Vmax) of the HATS (Wang *et al*., 1993). The regulation of gene expression in response to

N starvation has been studied in *Arabidopsis* for the multigenic *AtAMT* family (Gazzarrini *et al*., 1999; Rawat *et al*., 1999; Shelden *et al*., 2001). *AtAMT1;1* mRNA levels increased markedly over a 2-day period after N removal, whilst *AtAMT1;2* and *AtAMT1;3* were less affected. The high affinity of AtAMT1;1 for NH_4^+, and its co-regulation with NH_4^+ influx, suggest that AtAMT1;1 is a good candidate for an important component of the HATS. When N-depleted plants were re-supplied with NH_4^+ or amino acid, feedback signals led to a rapid decrease of net NH_4^+ uptake in wheat (Glass and Siddiqi, 1995). The same was true for *Arabidopsis* (Rawat *et al*., 1999) and tomato (von Wiren *et al*., 2000) although gene expression studies provide evidence that the *AtAMT1* and the *LeAMT1* transporters are not regulated in the same way. Whilst *LeAMT1;1* and *AtAMT1;1* respond similarly by a decrease in mRNA levels, *LeAMT1;2* is induced in roots by NH_4^+, and even more strongly by NO_3^- supply (von Wiren *et al*., 2000). When tomato plants are grown under NO_3^- nutrition and low CO_2, the expression of *LeAMT1;1* and *LeAMT1;3* is slightly higher in leaves, suggesting that the corresponding protein could play a role in the retrieval of NH_4^+ derived from photorespiration. Gene expression was recently analysed in rice and revealed distinct N-dependent regulation for *AMTs*, differing from that in tomato or *Arabidopsis* (Sonoda *et al*., 2003).

Light and/or photosynthesis also controls NH_4^+ uptake. During a day/night cycle, NH_4^+ uptake peaks at the end of the light period and is induced by sugar during the dark phase. Again, this corresponds to the regulation of *AMT* gene expression in *Arabidopsis* (Gazzarrini *et al*., 1999), tomato (von Wiren *et al*., 2000) and tobacco (Matt *et al*., 2001). Both diurnal variations and response to sucrose induce the expression of *AtAMT1;2* and *AtAMT1;3* which showed a more pronounced response to both signals than *AtAMT1;1* (Lejay *et al*., 2003). In addition to transcriptional regulation of NH_4^+ uptake, several lines of evidence also point to the possibility of post-transcriptional control. Using L-methionine-DL-sulfoximine (MSX) to block NH_4^+ assimilation, Rawat and colleagues demonstrated a 30% decrease in NH_4^+ influx rates without any decline in *AtAMT1;1* transcript levels (Rawat *et al*., 1999). The role of NH_4^+ ion itself in post-transcriptional regulation of the HATS is supposed to take place via a direct inhibition of AMT transport activity or by inhibiting the synthesis of AMT proteins (Crawford & Forde, 2002).

1.2.4 Nitrate uptake and transport

Nitrate influx has been studied intensively at the physiological and molecular levels (Muller *et al*., 1995; Devienne *et al*., 1994). In contrast, NO_3^- efflux, which redirects a significant proportion of the absorbed NO_3^-, has been rarely studied. Nitrate influx is mediated by two distinct systems, the HATS and the LATS. When $[NO_3^-]_{ext}$ is low (<1 mM), the HATS mediates NO_3^- influx,

first, at a low rate, assuming that the plants have not been previously exposed to NO_3^-, and then at a higher rate, as evidenced by changes in Km and Vmax (Hole *et al*., 1990; Aslam *et al*., 1992; Kronzucker *et al*., 1995). These characteristics indicate that there are two components in the HATS, one which is constitutive (cHATS) and the other inducible (iHATS). When $[NO_3^-]_{ext}$ exceeds 500 μM, the non-saturable LATS system becomes evident. Electrophysiological studies have demonstrated that both the HATS and LATS are mediated by electrogenic 1 $NO_3^-/2H^+$ symporters (Glass *et al*., 1992).

1.2.5 Identification of genes coding for nitrate transporters

Two gene families encode proteins that are involved in either the low (*NRT1*) or the high (*NRT2*) affinity NO_3^- systems. These families share structural features but no homology at the amino acid level.

1.2.5.1 The NRT1 *family of transporters*

The first gene encoding a low-affinity NO_3^- transporter was cloned in *Arabidopsis* by isolating and characterising a chlorate resistant T-DNA insertion mutant *chl1* (Tsay *et al*., 1993). Chlorate is an analogue of NO_3^-which is reduced to toxic chlorite by NR (see Section 1.3.1). *chl1* showed reduced NO_3^- uptake, particularly when plants were grown in the presence of NH_4^+ (Huang *et al*., 1996; Touraine & Glass, 1997). The corresponding *AtNRT1.1* cDNA encodes a 590-amino acid protein, containing 12 putative membrane-spanning domains. When expressed in *Xenopus* oocytes, this cDNA allowed NO_3^- uptake (Tsay *et al*., 1993) with biphasic kinetics (Liu *et al*., 1999). The dual affinity of the AtNRT1.1 transporter has since been shown to be regulated by a phosphorylation/de-phosphorylation mechanism (Liu & Tsay, 2003). Further, three other *AtNRT1* genes have since been identified in *Arabidopsis*, *AtNRT1.2*, *AtNRT1.3* and *AtNRT1.4*, which show 36%, 51% and 42% identity, respectively at the amino acid level with *AtNRT1.1*. Functional analysis of *AtNRT1.2* in *Xenopus* oocytes showed that it is also a low-affinity (Km = 6 mM) NO_3^- transporter (Liu *et al*., 1999). The functions of the two other genes are still not known. Another member of this family, *AtPTR2B*, encodes a peptide transporter (Rentsch *et al*., 1995; Song *et al*., 1996). Oligopeptide transport seems to be a feature of the NRT1 family as *BnNRT1.2*, which was one of the two cDNAs identified in *Brassica napus*, is also able to transport NO_3^- and L-histidine when expressed in oocytes (Zhou *et al*., 1998). Using *AtNRT1.1* as a heterologous probe, Lauter and colleagues have isolated two cDNAs from a tomato root-hair specific library (Lauter *et al*., 1996). Although the corresponding protein shares 65% identity with AtNRT1.1, their role in NO_3^- uptake remains to be demonstrated. Corresponding homologous genes have also been identified in *N. plumbaginifolia* (Fraisier *et al*., 2001).

1.2.5.2 The NRT2 *family of transporters*

Chlorate has also been used to screen for mutants affected in the HATS, but to date this has only been successful in fungi. In *Aspergillus nidulans*, the chlorate resistant *crna* mutant was shown to be defective in NO_3^- uptake. The *CRNA* cDNA encodes a transport protein of 507 amino acid containing 12 membrane-spanning domains with two groups of 6 segments separated by a central loop (Unkles *et al*., 1991). Two *CRNA*-related genes have since been isolated from *Chlamydomonas reinhardtii*: *CrNRT2.1*, which encodes a high affinity NO_3^-/NO_2^- bi-specific transporter, and *CrNRT2.2*, which encodes a high affinity NO_3^- specific transporter. The presence of a third protein, Nar2, was found to be necessary to form an active NO_3^- transport system (Quesada *et al*., 1994; Galvan & Fernandez, 2001).

In higher plants, the first *NRT2* genes were cloned in barley (Trueman *et al*., 1996) and *N. plumbaginifolia* (Quesada *et al*., 1997) by PCR amplification using degenerate primers corresponding to conserved motifs found in a subgroup of the major facilitator superfamily (MSF) transporters. Independently, the *AtNRT2.1* gene was subsequently isolated using differential display (Filleur & Daniel-Vedele, 1999) and PCR amplification (Zhuo *et al*., 1999) techniques. *NRT2* genes have since been identified in many other plants species (Fig. 1.2). The complete genome sequence of *Arabidopsis* has revealed the presence of seven *NRT2* genes, distributed across three chromosomes (Orsel *et al*., 2002a). *AtNRT2.1*/*AtNRT2.2* and *AtNRT2.3*/*AtNRT2.4* are arranged in tandem at the top of chromosome 1 and the bottom of chromosome 5, respectively, whilst *AtNRT2.6* and *AtNRT2.7* are located on chromosomes 3 and 5. Using the amino acid sequence of AtNRT2.1 as a reference, AtNRT2.2, AtNRT2.3, AtNRT2.4, AtNRT2.5, AtNRT2.6 and AtNRT2.7 proteins exhibit 91%, 77%, 88%, 69%, 77% and 57% similarity, respectively. A phylogenetic tree (Fig. 1.2) of all *Arabidopsis* and other higher plant sequences show that AtNRT2.1, AtNRT2.2, AtNRT2.3, AtNRT2.4 and AtNRT2.6 proteins are similar, whilst AtNRT2.5 and AtNRT2.7 are closer to lower eukaryotic (alga or fungi) than to other plant proteins. In contrast to *NRT1*, the only *NRT2* cDNAs that have been shown to mediate active NO_3^- uptake following injection into *Xenopus* oocytes are *CRNA* or *CrNRT2*. Further, the co-injection of *Nar2* with *CrNRT2.1* is required to obtain active NO_3^- uptake (Zhou *et al*., 2000).

Reverse genetics is a valuable part of the functional genomics toolkit since it allows the function of specific genes to be disrupted (Bouchez & Hofte, 1998). In *Arabidopsis*, extensive populations mutagenised with an insertion element (transposon or T-DNA) have recently become available (Bouche & Bouchez, 2001). A T-DNA mutant affected in both *AtNRT2.1* and *AtNRT2.2* genes has been identified, in which the HATS but not the LATS activities are disrupted (Filleur *et al*., 2001). This mutant could be used to determine the function of *NRT2* genes in global NO_3^- transport processes in plants. The organ specificity

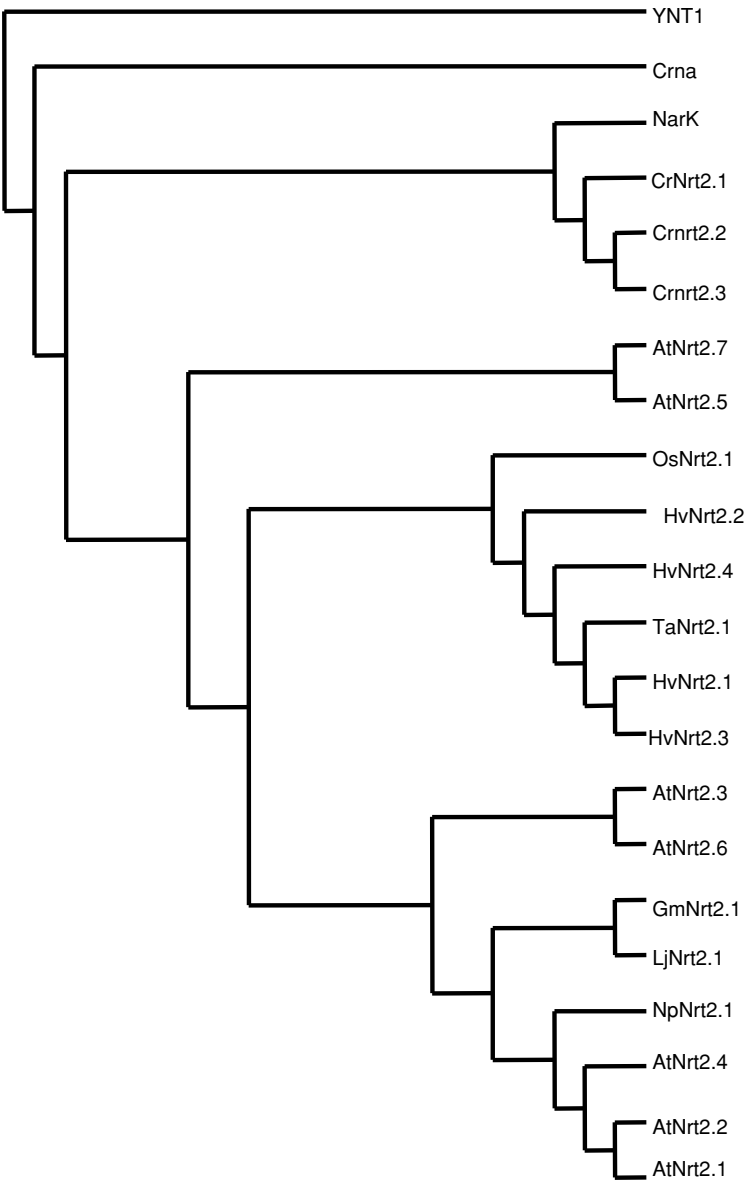

Figure 1.2 Unrooted tree of NRT2 proteins. Sequences are from *Hansenula polymorpha* (*YNT1*, NCBI protein number CAA93631), *Aspergillus nidulans* (*Crna*, NCBI AAA62125), *Escherichia coli* (*NarK*, NCBI CAA34126), *Chlamydomonas reinhardtii* (*CrNrt2.1*, NCBI CAA80925 ; *CrNrt2.2*, NCBI CAA80926; *CrNrt2.3*, NCBI CAA11238), *Arabidopsis thaliana* (*AtNrt2.1*, NCBI ACC64170; *AtNrt2.2*, NCBI AAC35884; *AtNrt2.3*, NCBI BAB10099; *AtNrt2.4*, NCBI BAB10098; *AtNrt2.5*, NCBI AAF78499; *AtNrt2.6*, NCBI CAB89321; *AtNrt2.7*, NCBI CAB87624), *Oryza sativa* (*OsNrt2*, NCBI BAA33382), *Hordeum vulgare* (*HvNrt2.1*, NCBI AAC49531; *HvNrt2.2*, NCBI AAC49532; *HvNrt2.3*, NCBI AAD28363; *HvNrt2.4*, NCBI AAD28364), *Triticum aestivum* (*TaNrt2*, NCBI AAK19519), *Glycine max* (*GmNrt2*, NCBI AAC09320), *Lotus japonicus* (*LjNrt2.1*, NCBI CAC35729), and *Nicotiana plumbaginifolia* (*NpNrt2.1*, NCBI CAA69387).

of expression also indicates the possible roles of *NRT2*. In higher plants, most *NRT2* genes isolated thus far are expressed preferentially in roots. In tomato, *LeNRT2* expression is not observed in whole shoots or leaves (Ono *et al*., 2000) whilst in *N. plumbaginifolia*, *NpNRT2.1* transcripts are detectable at low levels in leaves, petioles, buds flowers or seeds (Quesada *et al*., 1994). In *Arabidopsis*, Orsel *et al*. (2002b) have demonstrated variation in the expression levels between the seven genes within the *NRT2* gene family. However, although most of the *NRT2* genes are expressed more in roots than in shoots, *AtNRT2.7* showed a greater expression in the aerial tissues, which could indicate a role in NO_3^- fluxes within the leaves.

1.2.6 Regulation of nitrate influx and the role of NRT1 *and* NRT2 *genes*

The regulation of NO_3^- uptake is highly complex and it has been the subject of several reviews (Crawford & Glass, 1998; Daniel-Vedele *et al*., 1998; Forde & Clarkson, 1999; Forde, 2000; Galvan & Fernandez, 2001; Glass *et al*., 2001; Williams & Miller, 2001). Both environmental factors and internal signals control NO_3^- uptake mediated by HATS and LATS. As indicated previously, NO_3^- itself is an inducer, which discriminates between constitutive (cHATS and cLATS) and inducible (iHATS) NO_3^- uptake systems (Behl *et al*., 1988). As opposed to NO_3^-, addition of reduced N sources such as NH_4^+ or amino acids to the culture medium inhibits NO_3^- uptake (Muller & Touraine, 1992; Kronzucker *et al*., 1999). Nitrate uptake is also regulated by diurnal cycles and light intensity, which may be due to the transport of photosynthates to the root (Delhon *et al*., 1995). Internal signals are thought to match the rate of N acquisition to the demand for N (Glass & Siddiqi, 1995). During N starvation, plants increase their capacity to absorb NO_3^- transiently, which may be a consequence of de-repression of NO_3^- transport due to N metabolites accumulating under non-limiting conditions. After NO_3^- is re-supplied, feedback regulation takes place (Siddiqi *et al*., 1989), but the signals responsible for the decrease in NO_3^- influx have not yet been identified.

How does NO_3^- influx and gene expression correlate? In *Arabidopsis*, the expression of *AtNRT2.1* and regulation of NO_3^- influx are tightly linked. For example, *AtNRT2.1* is induced by low levels of NO_3^- to a transient maximum. Further, *AtNRT2.1* expression transiently induced by N starvation (Filleur & Daniel-Vedele, 1999; Zhuo *et al*., 1999) is strictly correlated to the influx during a day/night cycle and it is inducible by sugars (Lejay *et al*., 1999). The regulation of *AtNRT2.1* may depend on the C flux from glycolysis (Lejay *et al*., 2003). These correlations, together with defects of the regulation of iHATS activities (NO_3^- inducible, starvation de-repressible and NH_4^+ repressible high affinity uptake) in the *atnrt2a* mutant (Cerezo *et al*., 2001) strongly support the hypothesis that the *AtNRT2.1*/*AtNRT2.2* genes play a major role in the NO_3^- uptake mediated by the iHATS. The role(s) of other *AtNRT2* genes remains to